1 **Title:** The axon initial segment drives the neuron's extracellular action potential

2

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1 **Key points summary**

- 2 • The neuron's soma and dendrites are thought to give rise to its
- 3 extracellular voltage signal, while signals from the axon are usually
- 4 considered negligible.
- 5 • Instead, we found that the largest amplitude of the extracellular signal
- 6 originates from the axon initial segment, not from the soma.
- 7 • This finding shows that changes in the AIS position and function can be
- 8 observed in high spatiotemporal detail by means of high-density
- 9 extracellular electrophysiology.

10

11 **Abstract:** Extracellular voltage fields produced by a neuron's action potentials
 12 provide a primary means for studying neuron function, yet their biophysical
 13 sources remain ambiguous. The neuron's soma and dendrites are thought to
 14 drive the extracellular action potential (EAP), while the axon is usually ignored.
 15 However, by recording voltages of single neurons in dissociated rat cortical
 16 cultures and Purkinje cells in acute mouse cerebellar slices at hundreds of sites,
 17 we find instead that the axon initial segment dominates the EAP, and,
 18 surprisingly, the soma shows little or no influence. As expected, this signal has
 19 negative polarity (charge entering the cell) and initiates at the distal end.
 20 Interestingly, signals with positive polarity (charge exiting the cell) occur near
 21 some but not all dendritic branches and occur after a delay. Such basic
 22 knowledge about which neuronal compartments contribute to the extracellular
 23 voltage field is important for interpreting results from all electrical readout
 24 schemes. Moreover, this finding shows that changes in the AIS position and

1 function can be observed in high spatiotemporal detail by means of high-density
2 extracellular electrophysiology.

3 **Abbreviations:** ACSF, artificial cerebrospinal fluid; AIS, axon initial segment;
4 AnkG, ankyrin-G; CMOS, complementary-metal-oxide-semiconductor; DMEM,
5 Dulbecco's modified eagle medium; EAP, extracellular action potential; GAD67-
6 GFP, glutamic acid decarboxylase 67 - green fluorescent protein; MAP2,
7 microtubule-associated protein 2.

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10 **Introduction**

11 How different neuronal compartments and different cell types contribute to the
12 extracellular action potential (EAP) remains unclear and without sufficient direct
13 experimental evidence. A variety of theoretical (Rall, 1962; Holt and Koch, 1999;
14 Gold et al., 2006; Pettersen and Einevoll, 2008; Einevoll et al., 2013) and a limited
15 number of experimental (Grace and Bunney, 1983; Henze et al., 2000; Frey et al.,
16 2009; Buzsaki et al., 2012; Baranauskas et al., 2013; Anastassiou et al., 2015;
17 Petersen et al., 2015) studies address the subcellular origins of EAPs. In most,
18 axonal contributions to the EAP have been considered negligible or small,
19 possibly due to a notion that small cell compartments require less current to
20 depolarize (Rall, 1962; Humphrey and Schmidt, 1990). Along these lines, initial
21 patch-clamp data shows similar AIS and somatic sodium currents per area of
22 membrane (Colbert and Johnston, 1996), which are the currents responsible for
23 action potential generation. In other words, the much larger soma would
24 produce a much greater cumulative current. On the other hand,
25 immunohistological evidence shows that sodium channels are expressed to a

1 much high degree in the AIS (Hu et al., 2009). There, they are tightly coupled to
 2 the actin cytoskeleton and not easily drawn into a patch pipette, which suggests
 3 that the initial patch-clamp data may underestimate sodium current at the AIS
 4 (Kole et al., 2008). Recent quantitative freeze-fracture immunogold labeling
 5 (Lorincz and Nusser, 2010), outside-out patch clamp recordings (Hu et al., 2009),
 6 and experimentally constrained models (Kole et al., 2008) show that the AIS of
 7 pyramidal neurons has about 35 to 50 times higher sodium channel density than
 8 the soma or proximal dendrites. Likewise, while not focusing on the EAP, early
 9 modeling studies predict that a 20- to 500-fold higher density of sodium
 10 channels is necessary for action potentials to initiate in the AIS (Dodge and
 11 Cooley, 1973; Mainen et al., 1995). A simplistic calculation using a 50-fold
 12 increase shows that the AIS would indeed be able to influence the EAP: an AIS
 13 would have approximately 5 times greater total sodium conductance than a
 14 soma, assuming a 17- μ m diameter spherical soma and a 31- μ m length cylindrical
 15 AIS (mean values from our data, which also matches ratios in (Mainen et al.,
 16 1995)) with an estimated 1 μ m diameter; conductance is considered to be
 17 proportional to surface area multiplied by the channel density. However,
 18 whether or not the AIS has more sodium channels than the soma, and to what
 19 degree, remains debated (Colbert and Johnston, 1996; Kole et al., 2008; Schmidt-
 20 Hieber and Bischofberger, 2010; Hallermann et al., 2012).

21

22 Here, we compared neurons' EAPs to fluorescence images of cell
 23 morphology in primary rat cortical cultures and acute mouse cerebellar slice
 24 preparations and present direct evidence that the EAP is driven by the AIS, not
 25 the soma. The spatial distribution and strength of the EAP is related to the AIS's

geometry and degree of formation. Our results add experimental evidence into the debate about the distribution of sodium channels in a neuron. To gather the data, neurons' EAPs were recorded in high spatial detail by using custom microelectrode arrays containing 11,011 densely packed electrodes ($17.8\ \mu\text{m}$ pitch; $8.2 \times 5.8\ \mu\text{m}^2$ area per electrode (Frey et al., 2010)). Immediately after recording, cells were imaged in order to match which cell compartments contributed to the neurons' EAPs. For the primary cultures, spontaneous EAPs of single neurons were sampled at multiple locations simultaneously at 2 to 3 weeks in vitro; to minimize overlap of voltage signals in recordings and neurites in images between neighboring neurons, we grew low-density cultures that contained a few to tens of neurons per array. A GAD67-GFP knock-in mouse line (Tamamaki et al., 2003) that allowed live imaging of Purkinje cells was used for acute slice recordings. We conclude with a brief discussion about how our results can affect interpretations of extracellular data.

Methods

Animal use. Protocols were approved by the Basel Stadt veterinary office according to Swiss federal laws on animal welfare and the Animal Research Committee of the RIKEN Center for Developmental Biology.

Low-density cell culturing. Cells from embryonic day 18 Wistar rat cortices were dissociated in trypsin, followed by mechanical trituration, and 1000 cells were seeded over an area of $\sim 4\ \text{mm}^2$ on top of the microelectrode arrays. Neurons and glia grew in 2 mL of serum-containing Neurobasal-based media for 5 days. Thereafter, half of the media was changed once a week to serum-

1 containing DMEM-based media. Detailed media recipes are in Bakkum et al.,
2 2013.

3

4 **Immunocytochemistry.** The neural source of extracellular signals was verified
5 optically by immunocytochemistry performed immediately after a recording
6 session. A detailed protocol is given in (Bakkum et al., 2013). The primary
7 antibodies to microtubule-associated protein 2 (MAP2; Abcam ab5392;
8 RRID:AB_2138153) and ankyrin-G (AnkG; NeuroMab clone N106/36;
9 RRID:AB_10697718) were diluted 1:200 and left overnight at 4°C on a shaker set
10 to low speed. The secondary antibodies containing Alexa Fluor 647
11 (RRID:AB_10374876) or Alexa Fluor 488 (RRID:AB_10561551) were diluted
12 1:200 and left for 1 hour at room temperature in the dark.

13

14 **Acute cerebellar slice preparation.** GAD67-GFP knock-in mice (Tamamaki et
15 al., 2003), postnatal day 21-37, were obtained from the Laboratory for Animal
16 Resources and Genetic Engineering in RIKEN Center for Developmental Biology
17 Kobe, Japan. The mice were anaesthetized by isoflurane inhalation and then
18 decapitated. The procedure for preparing cerebellar slices has been adapted
19 from (Frey et al., 2009). Briefly, the brain was dissected and immediately
20 immersed in ice-cold carbogenated artificial cerebrospinal fluid (ACSF)
21 containing in mM: NaCl 125, KCl 2.5, NaH₂PO₄ 1.25, MgSO₄ 1.9, Glucose 20,
22 NaHCO₃ 25. The cerebellum was separated from the cortex by cutting with a
23 blade and glued onto a tray along its sagittal plane. Parasagittal cerebellar slices
24 (150-200 µm thick) were obtained using a vibratome (Leica VT1200S). The slices

1 were incubated at 35°C in ACSF with 2 mM CaCl₂ for 30-45 minutes and then
2 maintained at room temperature until recording.

3

4 **Recording EAPs with CMOS-based microelectrode arrays.** Cortical networks
5 were grown for two to three weeks over 11,011-electrode complementary-
6 metal-oxide-semiconductor-based (CMOS) microelectrode arrays (1.8 × 2.0 mm²
7 area containing 8.2 × 5.8 μm² electrodes; 17.8 μm electrode pitch; 20 kHz
8 sampling rate). Acute cerebellar slices were placed flat on the arrays and
9 continuously superfused with carbogen-loaded ACSF (~30-33°C) throughout the
10 experiment. See (Frey et al., 2010) for circuit details of the CMOS-based
11 microelectrode arrays. To identify the locations of the neurons growing over the
12 array, a sequence of overlapping recording configurations was scanned across
13 the array. A configuration is defined as how switches are set to connect a subset
14 of the electrodes to the 126 available readout channels. Switches are re-
15 configurable within a few milliseconds. The largest possible contiguous block
16 includes 6x17 electrodes, and 147 overlapping block configurations were used to
17 scan the whole array at a rate of 1 min per configuration. A recording session
18 lasted 2.5 hours. Recordings were acquired inside an incubator in order to
19 control environmental conditions (36°C and 5% CO₂).

20

21 **Spatial and temporal upsampling.** Signals were up-sampled to 160 kHz using
22 the Nyquist-Shannon sampling theorem and the Whitaker-Shannon interpolation
23 formula to improve temporal resolution (Blanche and Swindale, 2006). The
24 theorem is a mathematical proof that any sampled signal can be perfectly
25 reconstructed into the original analog signal, as long as the analog signal has

1 frequency components less than the Nyquist frequency. The microelectrode
2 array circuitry filters the analog data with a first-order filter with an upper cutoff
3 frequency of 3.7 kHz before data is sampled at 20 kHz, which satisfies the
4 requirements. To estimate waveforms between recording electrodes, up-
5 sampled and averaged EAPs were then spatially interpolated across a 1x1 μm
6 grid by cubic interpolation in Matlab ('griddata' function; MathWorks Matlab
7 R2014a).

8

9 **Statistics.** Pearson correlation and two-side t-tests were done in Matlab R2014a
10 (MathWorks).

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13 **Results**

14 The largest extracellular spike always had negative polarity (Figure 1B) and was
15 typically localized near the proximal AIS or the peak Ankyrin-G (AnkG; a
16 molecular marker of the AIS) immunofluorescence signal (Figure 1A) but never
17 at the soma (Figure 1C). Especially, the signals of cells whose AIS originated from
18 a dendrite (e.g. Figure 1A bottom), clearly evidenced that the EAP was correlated
19 with AIS location and shape. Signals near the soma of such cells typically were
20 not detectable unless averaging multiple EAPs (Figure 1B bottom, electrode 5).
21 An AIS was considered to have a dendritic origin if it began at a neurite branch
22 point, whose other branch expresses microtubule-associated protein 2 (MAP2; a
23 molecular marker of somatodendritic regions) and not AnkG. Out of the 49
24 neurons from 12 cultures (neurons that may have had overlapping voltage
25 signals were excluded), 26 had an AIS with a dendritic origin, which is a common

phenomenon observed in a variety of preparations (Triarhou, 2014). As an 'axon hillock' is not obvious for such cells, we define the 'neck' as the distance from the proximal end of the AIS to the soma. The neck length was $31 \pm 24 \mu\text{m}$ (mean $\pm$ s.d., $N = 26$) for AISs of dendritic origin and $7 \pm 7 \mu\text{m}$ ($N = 23$) for AISs of somatic origin. A significant relationship did not exist between the largest EAP spike and neck length (Pearson's correlation coefficient $r = 0.16$, $p = 0.27$, $N = 49$). Moreover, EAP spike magnitude did not depend on whether the AIS was of somatic or dendritic origin ($p = 0.71$, two-side t-test, $N = 49$), which demonstrated that the relative location of the soma does not strongly influence the magnitude of the EAP. Importantly, AIS lengths were positively correlated to spike magnitude ($r = 0.28$, $p = 0.05$, $N = 49$). Soma diameter was positively correlated to the largest EAP spike for AIS with somatic ($r = 0.47$, $p = 0.02$, $N = 23$) but not dendritic ($r = 0.06$, $p = 0.75$, $N = 26$) origins. Overall, AIS lengths and soma diameters were $31 \pm 13 \mu\text{m}$ and $17 \pm 5 \mu\text{m}$, respectively (mean $\pm$ s.d., $N = 49$).

Consistent with most reports in the literature, action potentials typically initiated at the distal AIS (Hu et al., 2009; Baranauskas et al., 2013), and EAP magnitude at the distal AIS was lower than the largest EAP (Figures 1C, 2, 3A-D). Dual-patch recordings of soma and axon (Yu et al., 2008; Baranauskas et al., 2013), voltage sensitive dye recordings (Palmer and Stuart, 2006), and modeling (Kole et al., 2008; Baranauskas et al., 2013) show that action potentials initiate in the axon at about $40 \mu\text{m}$ from the soma. This is consistent with the location of the distal end of the AIS we found for AISs originating from the soma ($36 \pm 13 \mu\text{m}$, mean $\pm$ s.d.). Once initiated, action potentials propagated in both directions: into

1 the axon proper and backwards through the proximal AIS and towards the soma.
2 This scenario holds for both cases of axons originating directly from the soma or
3 from dendrites (Figure 2). Of the 22 neurons with an AIS length longer than 30
4 μm , the propagation velocity was 1.1 ± 1.5 m/s (mean $\pm$ s.d.; 65.6 μm and 0.6 m/s
5 median); the distal end of the AIS always fired first. The velocities were similar to
6 those of action potentials propagating in unmyelinated cortical axons (Bakkum
7 et al., 2013). Some of the neurons with shorter AIS lengths showed action
8 potentials occurring nearly simultaneously throughout the AIS or, in 2 cases, first
9 at the proximal end of the AIS or at the soma (Figure 3D). For the 15 neurons
10 with a neck length greater than 30 μm , the propagation velocity from the
11 proximal AIS to the soma center was slower at 0.3 ± 0.2 m/s (mean $\pm$ s.d.; 0.2
12 m/s median).

13

14 As a control, live-cell imaging and patch clamp electrophysiology verified
15 that the largest-amplitude extracellular action potential is spatially offset from
16 the soma and distally initiated (Figure 3E-F). Here, action potentials were evoked
17 by applying current injection through the patch pipette. Results were consistent
18 across five experiments. Alexa Fluor 594 dye was injected into the cell via the
19 patch pipette in order to perform simultaneous live-cell imaging. The
20 extracellular signal is approximately proportional to the negative derivative of
21 the intracellular signal (Henze et al., 2000) (and is proportional to the membrane
22 current) (Henze et al., 2000; Gold et al., 2006; Einevoll et al., 2013). Then the
23 peak negative extracellular amplitude corresponds to the point of maximum
24 positive slope on the intracellular trace (Figure 3F). The EAP is ~ 2 orders of
25 magnitude smaller than an intracellular AP. Therefore, periods of slower change

1 in the intracellular AP correspond to a small or not detectable EAP. The initial
2 positive deflection in the EAP near the soma is typical of capacitive membrane
3 currents. These would be caused by intracellular currents, spreading axially from
4 the AIS, charging the membrane prior to the opening of voltage gated ion
5 channels.

6
7 While the largest amplitude spikes had negative polarity and were co-
8 localized with AISs, spikes with positive polarity were co-localized with some,
9 but not all, dendritic branches (Figure 4A). The average peak negative spike was
10 6.9 times bigger than the average peak positive spike and 5.9 times bigger than
11 the average somatic spike, which also had a negative polarity (N = 24) (Figure
12 4C). The co-localization of the positive peaks with dendrites and their mostly
13 monophasic nature suggest that they are caused by a passive (i.e., capacitive)
14 return current balancing the influx of sodium ions at the AIS. Alternatively,
15 voltage-gated potassium channels are expressed in dendrites and could also be
16 involved (Kornegreen and Sakmann, 2000; Martina et al., 2003). Interestingly, in
17 our measurements (Figure 4B) we found a delay between the occurrence of
18 negative and positive spikes: $113 \pm 108 \mu\text{s}$ (mean $\pm$ s.d., $86 \mu\text{s}$ median, N = 24; see
19 also Movie 1). The delay was significantly correlated to axial distance ($r = 0.79$, p
20 $= 0.001$; Figure 4B) and not straight-line distance ($r = 0.34$, $p = 0.11$), suggesting
21 an intracellular influence (Destexhe and Bedard, 2012). An ideal return current
22 would result in simultaneous peaks. However, signals at these locations will be
23 influenced by other components, such as active ionic currents from nearby
24 neurites and the presence of other cells, such as glia, that may affect resistivity.
25 Likewise, how and why dendritic branches differentially contribute to the EAP

(Figure 4A) is an important future study; possibly, the intracellular resistance varies for different dendritic branches. For Figure 4, 25 of the 49 neurons were excluded because either some distal parts of their dendritic arbor were not recorded at the same time as the AIS or their dendritic arbor overlapped with neurites from another cell.

6

To investigate the role of the AIS also in neuronal tissue closely resembling in-vivo morphology, we performed further experiments in Purkinje cells in acute cerebellar mouse slices. Purkinje cells differ from cortical neurons by having a more passive dendritic tree and larger soma. These two features would make it more likely that the largest extracellular signal would arise from the soma, if the soma instead of the AIS would be the source of the signal. Nevertheless, corroborating the results from cortical cell cultures above, the largest negative EAPs (Figure 5A) occurred at the area containing axons instead of at the Purkinje cell layer (i.e., the location of all Purkinje somas), and the positive EAPs (Figure 5B) consistently occurred in the area containing dendrites. In all cases, the largest negative EAPs were found to co-localize with the putative location of the Purkinje cell AISs; the mean of negative amplitudes peaked sharply approximately 40 μm away from the center of the Purkinje cell layer, in the granular cell layer (Figure 5C). On the other hand, the largest positive EAPs (i.e., the return currents) occurred 40 to 100 μm away from the center of the Purkinje cell layer, in the molecular layer. Purkinje cells have a stereotypical orientation: their AISs are always at the granular cell layer, between the white matter and the Purkinje cell layer; their dendritic arbors are located in the molecular layer, fanning out opposite the AIS from the Purkinje cell layer

1 towards the pia. Parasagittal slices were made in order to keep the full
2 morphology of Purkinje cells intact for recording. Purkinje cells are GABAergic
3 and produce large spontaneous spikes even without synaptic input; the parallel
4 fibers that would provide input get cut during slicing.

5

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7 **Discussion**

8 Extracellular signal amplitudes can be influenced by many factors, such as
9 electrode impedance, the local microenvironment, or the degree of coupling
10 between a neuronal membrane and an electrode. However, these influences
11 likely had a negligible effect on our results. We deposited platinum black on the
12 electrodes (see Methods). Besides reducing the electrode impedance, platinum
13 black also reduces variations in signal amplitude due to impedance mismatches
14 across the arrays' electrodes to about 5% (Viswam et al., 2014). This variation is
15 negligible when compared to the AIS-to-soma or AIS-to-dendrites signal
16 amplitudes that differ by over 600% on average (Fig. 4C). Consistent results
17 across 49 individual neurons and multiple preparations provide strong evidence
18 that the results were not an artifact from variations in local microenvironments
19 or coupling. Further evidence is that a neuron's EAP is detected by multiple
20 neighboring electrodes, including sites that are not immediately adjacent or
21 coupled to a neuronal membrane, and that the signal attenuates with increasing
22 distance from the source as expected (Figs. 1A, 2A, 3E, 4A).

23

24 The general phenomenon that the largest signal amplitudes occur in the
25 region of the AIS is applicable to both in vitro and in vivo situations. The EAP

1 arises from neuronal membrane currents, in particular from voltage-gated
 2 sodium channels. Previous studies showed that the molecular composition of
 3 axons in vitro and in vivo, including sodium channel distribution in the AIS, are
 4 consistent (Hedstrom et al., 2007). This implies that the membrane currents
 5 responsible for the EAP will likewise be similar. In turn, how the electric field
 6 spreads in the medium and is picked up as a potential on the recording
 7 electrodes is governed by laws of physics, i.e., Maxwell's equations (Einevoll et
 8 al., 2013). In other words, this aspect is independent of whether the
 9 experimental preparation is in vitro or in vivo. Nevertheless, we also
 10 experimentally addressed this issue and found consistent results in acute
 11 cerebellar slices, which preserve in vivo local microarchitecture (Fig. 5).

12
 13 The basic and fundamental observation of the EAP being driven by
 14 currents from the AIS instead of the soma can affect how extracellular recordings
 15 are interpreted. For example, neurons with spherically symmetric dendrites are
 16 thought to produce small EAPs due to the summation of dendritic membrane
 17 currents canceling each other out (axonal contributions presumed negligible)
 18 (Logothetis, 2008; Buzsaki et al., 2012). However, such symmetry is broken
 19 when membrane currents originate from the AIS or from specific dendritic
 20 branches (Figure 4A). Likewise, neurons with smaller soma are thought to
 21 produce smaller EAPs (Humphrey and Schmidt, 1990), but we observed this only
 22 for neurons with an AIS originating from the soma. Why wide variations in EAP
 23 waveforms exist between cells has been unclear (Anastassiou et al., 2015) but
 24 can be explained to a degree by variations in AIS shape and level of AIS
 25 formation. In our preparation, EAP magnitudes ranged between 67 to 952 μ V (up

1 to 1.8 mV in previous data (Bakkum et al., 2013)) and AIS lengths between 13 to
 2 71 μ m. ‘Silent’ cells existed and were typically associated with decreased or no
 3 AnkG expression. Occasionally, AISs were forked, which produced distorted or
 4 double-spiked EAPs depending on the recording location. Why different
 5 dendritic branches produce different extracellular signals (cf. Figure 4A) is
 6 harder to explain and may require different experimental approaches to
 7 decipher. In tangential work, the AIS has been identified as the site with the
 8 lowest threshold and highest reliability for extracellular stimulation (McIntyre
 9 and Grill, 1999; Fried et al., 2009; Radivojevic et al., 2016), whereas stimulation
 10 under the soma often failed to evoke action potentials (Radivojevic et al., 2016).
 11 This finding supports the AIS's higher contribution to the EAP and provides
 12 further indirect evidence that the AIS contains a higher concentration of voltage-
 13 gated ion channels. In summary, the action's at the AIS.

14

Additional information

16 **Competing Interests:** M.E.J.O. and U.F. are co-founders of MaxWell Biosystems
 17 AG, Mattenstrasse 26, c/o ETH Zurich, Basel 4058, Switzerland.

18

19 **Author Contributions:** Experimental design and data analysis was done by
 20 D.J.B., D.J., and M.E.J.O.; D.J.B., D.J., M.E.J.O., and M.R. performed experiments;
 21 technical support was provided by D.J.B., D.J., U.F., and M.R.; D.J.B., U.F., M.E.J.O.,
 22 A.H., and H.T. wrote the manuscript; and project supervision was done by U.F.,
 23 H.T. and A.H. All authors approve the final version of the manuscript and agree to
 24 be accountable for all aspects of the work in ensuring that questions related to
 25 the accuracy or integrity of any part of the work are appropriately investigated

1 and resolved. All listed authors qualify for authorship, and all those who qualify
2 for authorship are listed.

3

4 **Funding:** This work was supported by the European Community through the
5 European Research Council Advanced Grants 267351 'NeuroCMOS' (FP7) and
6 694829 'neuroXscales' (Horizon 2020), the Swiss National Science Foundation
7 Grant 205321_157092/1, KAKENHI Grant (16K14191, 26630089, and 2570183),
8 Kayamori Foundation of Informational Science and Advancement, and The Asahi
9 Glass Foundation. The funders had no role in study design, data collection and
10 analysis, decision to publish, or preparation of the manuscript.

11

12 **Acknowledgments:** We thank Alexander Stettler and Albert Martel for post-
13 processing CMOS chips; Felix Franke and Wei Gong for critical discussions; Jan
14 Muller for technical support; and the D-BSSE staff for expediting experiments.

15

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1 **Figure Legends**

2

3 **Figure 1.** Neurons' negative-amplitude extracellular action potentials co-localize
 4 with their AISs. **A.** Spatial and temporal (color) distribution of the averaged
 5 spontaneous extracellular action potential for two neurons. Dots are recording
 6 electrode locations, and dot sizes represent the AP negative peak voltage in the
 7 EAP. The underlying fluorescence images show somas and dendrites (black;
 8 MAP2) and the AIS (red; AnkG). The EAPs are the average of 14 (top neuron) and
 9 200 (bottom) spontaneous spikes. Numbered electrodes correspond to those in
 10 B. Scale bar: 20 μ m. **B.** Raw voltage waveforms for a single action potential (left)
 11 and the averaged waveform as used in A (right; N = 14 top and 200 bottom).
 12 Colored dots correspond to those for the same electrodes numbered in A. Scale
 13 bars: 100 μ V and 5 ms. **C.** Summary statistics of negative spike heights. Data in
 14 the bar plots (mean $\pm$ s.d., N = 49) are normalized by the peak voltage amplitude
 15 in the EAP.

16

17 **Figure 2.** Detailed EAP. **A.** Spatial and temporal distribution of the averaged
 18 spontaneous EAP for a neuron with an AIS originating from a dendrite (top) and
 19 an AIS originating from a soma (bottom). Dots mark recording-electrode
 20 locations. Time (dot color) is the delay from the first voltage peak, detected at the
 21 distal AIS, until the peak absolute voltage signal (dot size) at each location.
 22 Axonal and somatic areas correspond to signals that had larger negative peaks,
 23 while the dendritic area corresponds to signals that had larger positive peaks.
 24 The underlying fluorescence image shows somas, dendrites (black; MAP2), and
 25 the AIS (red cartoon; AnkG). The inset fluorescence image shows MAP2 (dark)

1 and AnkG (light); dark rectangles are electrodes. Scale bar: 50 μm . **B.** Average
2 voltage waveforms recorded at the electrodes with matching labels to those in A
3 (i.e. numbers and letters). Arrows indicated signal propagation direction. Scale
4 bars: 100 μV vertical; 1 ms horizontal.

5

6 **Figure 3.** Action potentials typically initiated at the distal AIS. **A, B, C.** Left:
7 Spontaneous action potentials at the distal AIS (blue), peak ankyrin-G
8 fluorescence signal (blue-green), proximal AIS (green), and soma (black).
9 Vertical lines indicate the timing of the negative voltage peak voltage. Waveforms
10 are the average of 11, 14, and 69 spikes for A, B, and C. Right: Silhouettes of
11 immunostained neurons' fluorescence images (black: MAP2, red: ankyrin-G).
12 Colored arrows and the white dot indicate the location of the plotted waveforms.
13 Scale bars: 100 μV vertical; 100 μs left; 20 μm right. **D.** Summary statistics. Bar
14 plots are mean $\pm$ s.t.d, N = 49. **E.** Spatial and temporal (color) distribution of the
15 averaged EAP (dot sizes). The shaded area is a patch pipette that evoked action
16 potentials by current injection and delivered the fluorescent dye for live-cell
17 imaging. The labeled electrodes correspond to those in B: the 1st electrode
18 recording an action potential, the electrode recording the Peak voltage signal,
19 and the electrode nearest the Soma. Scale bar: 20 μm . **F.** Average intracellular
20 (gray) and extracellular (color) action potential waveforms (39 spikes). Colored
21 vertical lines indicate the timing of the negative voltage peak.

22

23 **Figure 4.** Positive-amplitude extracellular action potentials co-localized with
24 some, but not all, dendritic branches and occurred after the AIS spike. **A.** Contour
25 plots of the minimum (blue) or maximum (red) EAP within $\pm 500 \mu\text{s}$ of the

1 negative peak (black silhouettes: MAP2, red: ankyrin-G). The contours are
2 normalized to the largest negative signal (blue-to-green) or the largest positive
3 signal (red-to-yellow). The blue, red, and white (soma) dots are the locations of
4 the plotted waveforms. The two neurons in the top row are also in Figure 3.
5 From left to right and top to bottom, EAPs are averages of 11, 69, 28, 68, 52, and
6 199 spontaneous spikes. Scale bars: 100 μ V vertical; 1 ms right; 20 μ m left. **B.**
7 The time delay versus axial distance between the largest negative peak (blue)
8 and the largest positive peak (red) voltages in the EAP. The line is a linear
9 regression (Pearson's correlation coefficient $r = 0.79$, $p = 0.001$, $N = 24$). **C.**
10 Average peak negative, peak positive, and somatic spike amplitudes (mean $\pm$ s.d.,
11 $N = 24$). Time courses of the EAPs are seen in Movie 1.

12
13 **Figure 5.** Purkinje cells' negative and positive EAPs co-localize with AISs and
14 dendrites. **A.** Spatial distribution of the most negative EAPs recorded from an
15 acute cerebellar slice. Dots show the locations of recording electrodes that detect
16 EAPs (color) below a -60 μ V threshold. EAP amplitudes are the mean voltage of
17 the 20 largest events during a 10 second recording. The underlying black-and-
18 white live-cell fluorescence image, taken immediately after recording, shows the
19 location of the cerebellar layers: white matter (WM), granular cell layer (GCL),
20 Purkinje cell layer (PCL), molecular layer (ML), and pia. The somas of the
21 Purkinje cells are in the PCL (highlighted for clarity; easier to see in the smaller
22 inset image). All Purkinje cell dendrites are in the ML, all AISs are in the GCL. The
23 microscopy image captured cells at the top layer of the acute slice, while the
24 EAPs were recorded from cells at the bottom layer. A sample cartoon of a
25 Purkinje cell is shown in black with a red line estimating the AIS location. Scale

1 bar: 100 μm . **B.** Same as A except for EAPs above a 25 μV threshold. **C.** Summary
2 of EAP amplitudes. Gray dots represent the largest negative or positive EAP
3 amplitudes detected at each electrode versus the distance of the electrode from
4 the PCL. Colored curves represent the mean for both negative (blue-green) and
5 positive (yellow-red) EAPs as measured by the respective electrodes. The
6 minimum in the curve representing the negative EAPs (dashed line at -40 μm)
7 co-localizes with the putative location of AISs (red line in the cartoon of a
8 Purkinje cell) in the GCL. The largest positive EAPs co-localize with the dendrites
9 in the ML. Representative spike shapes of the negative (blue) and positive (red)
10 EAPs recorded from the acute cerebellar slice are shown on the right.
11
12 Movie 1. The videos give contour plots of the EAP from neurons in Figure 4.
13 While voltages in Figure 4 are the peak values within a $\pm 500 \mu\text{s}$ time range,
14 voltages here are measured at the time indicated in the movie text. The
15 background is a MAP2 fluorescence image with a drawn red line to indicate the
16 AIS locations. Blue contours are negative voltages, and red contours are positive
17 voltages. Adjacent contour lines are separated by -50 μV (blue; starting from -10
18 μV) or +10 μV (red; starting from +10 μV). Scale bar: 20 μm .

Figure 1

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Figure 2

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Figure 3

Figure 4

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Figure 5